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(54) Title: METHOD FOR THE DETECTION OF RAS ONCOGENES, IN PARTICULAR THE K-RAS ONCOGENE

(57) Abstract

The invention relates to an oligonucleotide primer sequence for use in *in vitro* amplification, characterised in that said primer sequence is capable of creating a *Bst*X I restriction site overlapping codon (12) and/or an *Xcm* I restriction site overlapping codon (13) or a *Bce* 83I restriction site overlapping codon (61) of the wild-type *K-ras* oncogene, methods of using said primer sequences for detecting activating mutations in codons (12 and/or 13 and/or 61) of the *K-ras* oncogene and kits for performing the methods.

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METHOD FOR THE DETECTION OF RAS ONCOGENES, IN
PARTICULAR THE K-RAS ONCOGENE

The present invention relates to the detection of mutations in Ras oncogenes, and in particular in the K-ras gene.

Ras oncogenes are implicated in the development of a range of cancers. In particular somatically induced, activating mutations at defined positions in ras genes are believed to be important causative events in the process of tumorigenesis. Ras gene mutations occur in approximately 30% of human tumours, including cancer of the lung, thyroid, colon, rectum, pancreas and breast, and certain melanomas and leukaemias, although their incidence does vary according to tumour type. In addition, certain experimental tumour systems have been shown to be associated with activated ras genes. More specifically, mutations of the K-ras gene have been reported to be as high as 90% in carcinomas of the pancreas, 50% in adenocarcinomas of the lung and 40% in adenocarcinomas of the colon.

Activation of the ras oncogenes appears to be most frequently associated with mutations at position 12, although activation at other positions e.g. positions 13 and 61 is also commonly observed. Thus, in recent years a number of tests for the detection of ras mutations have been developed as a means towards clarifying their functional role in tumorigenic pathways, and indeed for their potential utility in the diagnosis and prognosis of cancer.

Primer-mediated restriction fragment length polymorphism (RFLP) analysis was developed as a rapid, non-radioactive method for fast and simple large-scale detection of mutant ras genes (Kahn et al., 1990, Amplifications, 4, 22-26). This method relies upon the polymerase chain reaction (PCR) for amplification of the ras sequences and upon the introduction of restriction

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rectal cancer. Thus, a novel and advantageous primer system has been designed, for use in amplification or RFLP-based ras mutation detection methods, which permit the detection of mutations at codons 12 and/or 13 of the K-ras gene. A further primer system permits detection of mutations at codon 61.

In one aspect, therefore, the present invention provides an oligonucleotide primer sequence for use in in vitro amplification, characterised in that said primer sequence is capable of creating a BstX I restriction site overlapping codon 12 and/or an Xcm I restriction site overlapping codon 13 of the wild-type K-ras oncogene.

The sequence of the wild type human K-ras gene is shown in Figure 1.

Mediation of the formation of restriction sites by the primers of the invention is achieved by the provision of a mismatch in the primer at a site, which together with bases present in the wild-type gene sequence, creates the desired restriction site.

A primer sequence according to the invention may thus contain a CCA substitution at the final base of codon 8 and the first two bases of codon 9 of the K-ras gene.

When such a primer is used to direct the in vitro amplification (e.g. by PCR) of a wild-type K-ras sequence, two restriction sites are created, one overlapping the first two, potentially activating, bases of codon 12 (and therefore specific for wild-type codon 12) and the other overlapping the first two, potentially activating bases of codon 13, (and therefore specific for wild-type codon 13). Mutations in the final base positions of codons 12 and 13 do not give rise to changes in the amino acid encoded, and hence are not

ras sequences mutant at activating positions in codon

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61 of the wild type K-ras gene. Thus for example, a primer may be used which creates a Bce 83I restriction site overlapping codon 61.

In a further aspect the invention therefore provides an oligonucleotide primer sequence for use in in vitro amplification, characterised in that said primer sequence is capable of creating a Bce 83I restriction site overlapping codon 61 of the wild-type K-ras oncogene.

Such a primer is advantageous since it covers all possible mutations in codon 61 and thus may be relied upon to give an accurate diagnosis. A representative primer for codon 61 may comprise a sequence corresponding to a C substitution at the second base of codon 60 of the K-ras gene.

Identifying different mutations at different positions may be therapeutically very advantageous since it may permit the targetting of the therapy to the particular cancer concerned. Thus for example, therapies may be directed against the particular mutation, eg. by immunotherapy which stimulates T-cells to kill cells carrying the identified K-ras mutation. Such immunotherapeutic techniques are described in WO92/14756 of Norsk Hydro AS.

Moreover, there is a value in determining not only the codon in which the mutation has occurred but also the actual mutation concerned. As mentioned above certain cancers are associated with particular substitutions and furthermore, for some cancers, such as pancreatic cancer, certain mutations give a better prognosis than others. In other words, the prognosis of a cancer may depend on the precise nature of the mutation. Consequently, determination of the sequence following identification of the affected codon is therefore also of clinical value.

In addition to the immunotherapy mentioned above, such targetted therapies, which the present invention

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coating of a polymer carrying one of such functional groups, eg. polyurethane together with a polyglycol to provide hydroxyl groups, or a cellulose derivative to provide hydroxyl groups, a polymer or copolymer of acrylic acid or methacrylic acid to provide carboxyl groups or an amino alkylated polymer to provide amino groups. US patent No. 4,654,267 describes the introduction of many such surface coatings. Alternatively, the support may carry other moieties for attachment, such as avidin or streptavidin (binding to biotin on the nucleotide sequence), DNA binding proteins (eg. the lac I repressor protein binding to a lac operator sequence which may be present in the starting molecule), or antibodies or antibody fragments (binding to haptens eg. digoxigenin on the nucleotide sequence). The streptavidin/biotin binding system is very commonly used in molecular biology, due to the relative ease with which biotin can be incorporated within nucleotide sequences, and indeed the commercial availability of biotin-labelled nucleotides, and thus biotin represents a particularly preferred means for immobilisation.

Where a solid phase amplification procedure is to be employed, the primer of the invention may additionally comprise a further mismatch(es) to create one or more additional restriction sites upstream of the potentially activating positions. Cleavage at such additional restriction sites may be used to detach the nucleotide sequences from the solid support in a quick and simple manner.

A preferred primer sequence according to the invention has the base sequence:

5' ACTGAATATA AACTTGTGGT CCATGGAGCT 3' and is designated herein primer 5K1.

Each of the bases A and C is a codon for the amino acid

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above, in the in vitro amplification of K-ras sequences in the samples under investigation (i.e. in which activating K-ras mutations are to be detected) using, for example methods as described by Kahn et al. (Supra). Such a sample, which may comprise, for example, blood, serum, urine, expectorate, ascites or other biological fluids, tissue biopsies (which may be fresh or fixed) or even stool samples, optionally appropriately treated using standard techniques to release nucleic acids, will generally contain predominating amounts of wild-type K-ras sequences and minor amounts of mutant, activated, K-ras sequences. Thus for example the nucleic acid isolation technique of our co-pending British Patent Application No. 9323305.4 filed on 11 November 1993 may be used. This involves boiling the sample and allowing it to cool and condense on and within a high surface area solid support.

Any of the in vitro amplification techniques well known and described in the literature may be used. PCR and its modifications will however generally be the principal method of choice. In the case of classical PCR, two primers are of course required, hybridising to opposing strands of the target DNA. The primer of the invention will be used in this regard as the 5', or upstream, amplification primer, and a 3', or downstream, amplification primer may be selected according to choice. In this first amplification step, the choice of 3' primer is not especially critical, as long as it is capable of annealing to the template with sufficient specificity to enable specific amplification. Suitable 3' amplification primers include for example:

5' TTATCTGTAT CAAAGAATGG TCCTGCACCA 3' (3K1)
5' TATTAAAACA AGATTTAC 3' (3K3)

The sequences and positions of primers 5K1, 3K1 and 3K3 mentioned above, with respect to the wild-type human

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type sequences have been cleaved and hence are inaccessible to further amplification.

Following this, second, "enriching" amplification step the amplification mixture may be subjected to detection of the mutant K-ras sequence. This may take place by restriction endonuclease digestion and RFLP analysis using the procedure of Kahn et al., 1990 (Supra) or other detection methods. Particular mention may be made in this regard of the detection method known as "detection of immobilised amplified nucleic acids" or DIANA, which is a particularly advantageous technique to be used (see WO90/11369).

In the DIANA detection system, a further PCR amplification step is effected using nested primers, that is a first pair of primers to amplify the target nucleic acid in a first series of cycles, and a second pair of primers hybridising between the first primer pair in a second series of cycles. The inner primers used in the second cycle carry, respectively, means for immobilisation to permit capture of the amplified DNA and a label or means for attachment of a label to permit recognition. The means for immobilisation may, for example, be a hapten such as biotin or digoxigenin while the means for attachment of a signal may include a different hapten or, in a preferred embodiment, a 5'-non-hybridising DNA sequence which is capable of binding to a DNA-binding protein (e.g. the lac operator) carrying an appropriate label. The immobilisation means may also be attached via a 5'-non-hybridising DNA sequence.

As a further check on accuracy, or as the primary detection method, the amplified fragments may be subjected to sequence analysis to verify the mutation, using known sequencing techniques. As mentioned above, this has advantages from a diagnostic point of view. The advantages of this method are further illustrated by the following examples which are provided.

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restriction enzyme cleavage.

Thus for example, the following 3' amplification primer may be used: 5' GAATGGTCCT CCACCAGTA TATGGATATT A 5' (designated herein primer 3K2). The mismatches at positions C and G respectively serve to create BstX I and Xcm restriction sites. Primer 3K2 and its relative position is also shown in Figure 2.

Where an additional, selective "enriching" amplification step is employed according to the invention, the "modified" internal control 3' primer, will generally only need to be used in the enriching amplification step.

It may also in certain cases be desirable to introduce further modifications to the method, in order for example to enhance specificity and/or sensitivity. Thus it may sometimes occur, that not all of the wild-type K-ras sequences will be digested in the enzyme cleavage step, and that some undigested wild-type sequences may remain. Also, mutated and wild-type strands may reanneal to form heteroduplexes which are not recognised by the restriction enzymes. To enhance specificity of the subsequent "enriching" selective amplification stage, additional amplification and restriction cleavage steps may be employed, using for example nested primers to further enhance specificity. This will be described in more detail in the Examples below, and is illustrated schematically in Figure 3. It has been shown that by using two restriction endonuclease cutting steps, sensitivity may be improved up to 1:100,000.

Further modifications include as mentioned above the use of solid supports to immobilise the amplification products. In this case, one or both of the amplification primers may be-provided with means for immobilisation e.g. biotin or haptens etc as described above. The use of such a solid phase system is advantageous in that it is cleaner, more efficient and

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In all the above kits, nucleotide bases will normally be supplied together with appropriate buffers.

The following Examples are given by way of illustration only with reference to the following Figures in which:

Figure 1 shows the nucleotide and corresponding amino acid sequence of the human cellular proto-oncogene K-ras (c-ki-ras2). Mutating hot spots are underlined;

Figure 2 shows the sequences of the primers used in the Examples and their positions with respect to the wild-type K-ras gene. The cleavage sites of the restriction endonucleases BstXI and XcmI are also shown;

Figure 3 shows a schematic representation of the steps involved in carrying out the K-ras mutation detection procedure of the present invention as described in Examples 1 and 2;

Figure 4 shows the results of electrophoresis of the amplification products of Example 2 in a 4% agarose gel containing ethidium bromide and analysed under UV light; and

Figure 5 shows the results of sequence analysis of the products of Example 2, 6% polyacrylamide gel. Panel 3A shows the sequence of the mutated products obtained from the needle biopsy (sample 3A); Panel 3B shows the sequence obtained from the same sample using the same procedure but without adding endonuclease (BstXI) in the two intermediate digestions of non-mutated amplification product.

Example 1

This sets out a general description of the performance of the method of the invention, to detect mutation in codon 12 of the K-ras gene, using a primer according to the invention.

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endonuclease (Xcm 1) specific for the sequence
CCANNNNNNGTT (N = G, T, A or C).

9a: BstX 1 cuts the product with a normal codon 12 but not the mutated product. For several reasons there may not be a complete cutting of the normal sequence.

9b: Xcm 1 cuts both the normal and mutated product since there is no mutation in codon 13. For several reasons there may not be a complete cutting of the product.

10a/b-13a/b: The digested product is used as template in an amplification identical to step 6. The mutated product is preferentially amplified because most of the normal product has been cut by the enzymes. The

endonuclease digestion is repeated to increase sensitivity.

14a/b: The digested product is used as template in an amplification using a biotinylated 5'primer (5K1-bio) and a modified 3'primer (3K2) containing both of the two restriction sites.

15a/b: An amplification product containing the modifications is produced.

16a/b: The products are digested with their respective endonucleases.

17a: The mutated product is cut only at the control site introduced by 3K2, while the normal product is cut at both sites.

17b: All of the product is cut at both sites since there is no mutation in codon 13.

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DNA preparation:

From cell-line:

DNA from SW480 is extracted (phenol/chloroform) and quantified using standard methods. 1 μ g of this DNA is used as positive control of mutation.

From paraffin blocks:

Two 5 μ m thick slides are cut of from each of the blocks. The microtom blade is washed twice in xylol and once in ethanol before each cutting.

The slides are added to 500 μ l micro-centrifuge tubes containing:

Bind & Wash buffer (Dynal, Norway), 400 μ l:

Tris-HCl 10 mM, pH 7.5

EDTA 1mM

NaCl 2.0 M

5K1-Bio 3 pmol

The tubes are incubated at 94°C for 5-10 minutes in a thermal cycler, and the contents mixed twice on a vortex-mixer for 20 seconds during this incubation.

When the tubes has been cooled to ambient temperature (for about 3 minutes), the liquid phase is pipetted off and mixed with 20 μ g of streptavidin coated paramagnetic beads (Dynabeads® M-280 streptavidin, Dynal, Norway).

The mixture is left at ambient temperature for 15 minutes.

When the mixture is cooled to ambient temperature, the liquid phase is pipetted off and mixed with 20 μ g of streptavidin coated paramagnetic beads (Dynabeads® M-280 streptavidin, Dynal, Norway).

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Thermal-cycling is performed in 200 μ l MicroAmp™ reaction tubes with the GenAmp™ PCR System 9600 (Perkin-Elmer, CT, USA) :

One cycle consists of 30 sec denaturation at 94°C, 1 min. annealing at 50°C and 2 min. elongation at 72°C. After the last cycle the tubes are kept for 8 min. at the elongation temperature.

Destruction of non-mutated amplification product:

5 μ l of the amplification product is digested with restriction endonuclease BstX 1 (New England Biolabs, MA, USA) in a total volume of 20 μ l at conditions recommended by the supplier.

5 μ l of the digested solution is used as template in the proceeding amplification step.

Amplification/Digestion procedure:

The first PCR (A1) consists of 15 cycles with 5K1 and 3K1 as primers and genomic DNA as template.

The amplification product is then digested with BstX 1 (A samples, B samples follow the same procedure but without the enzyme (BstX 1)) to destroy product that is not mutated in codon 12 for further amplification.

The digested product is used as template for 15 more cycles with 5K1 and 3K1 as primers (PCR A2).

A second BstX 1 digestion (A samples, B samples follow the same procedure but without the enzyme (BstX 1)) is performed to increase sensitivity.

The digested product is used as template for 35 cycles (PCR

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same procedure, but without adding endonuclease (BstX 1) in the two intermediate destructions of non-mutated amplification product. This illustrates how a weak non-informative mutated band is enhanced, and the background of the normal allele is removed.

An identical procedure using endonuclease Xcm 1 instead of BstX 1 allows detection of mutations in codon 13.

restriction site upstream of the restriction site overlapping codon 12 and/or codon 13 of the wild-type K-ras oncogene.

9. An oligonucleotide primer sequence as claimed in claim 8 wherein said sequence comprises the base sequence:

5' ACTGAATTTA AACTTGTGGT CCATGGAGCT 3'.

10. An oligonucleotide primer sequence as claimed in any one of claims 1 to 9 wherein the sequence is 22 to 30 nucleotides long.

11. Use of an oligonucleotide primer sequence as claimed in any one of claims 1 to 10 in an in vitro amplification-based method for detection of activating mutations in codons 12 and/or 13 and/or 61 of the K-ras oncogene.

12. A method of detecting activating mutations in codons 12 and/or 13 and/or 61 of the K-ras oncogene, said method comprising subjecting a sample containing the target K-ras DNA to be detected to one or more cycles of in vitro amplification using as an amplification primer, an oligonucleotide primer sequence as claimed in any one of claims 1 to 10, followed by restriction endonuclease digestion of wild-type K-ras sequences, using BstX I and/or Xcm I and/or Bce 83I and detecting the said amplified mutant K-ras sequences obtained.

13. A method as claimed in claim 12 wherein restriction endonuclease digestion is followed by one or more further cycles of in vitro amplification whereby mutant K-ras sequences containing said activating mutations are enriched.

14. A method as claimed in claim 12 or 13 wherein an additional primer is used for amplification.

15. A method as claimed in claim 14 wherein the sequence of the additional primer is selected from:

c) SSR and the kit additionally comprises a reverse transcriptase and a further DNA oligonucleotides primer sequence, both oligonucleotides being provided with a polymerase binding site; or

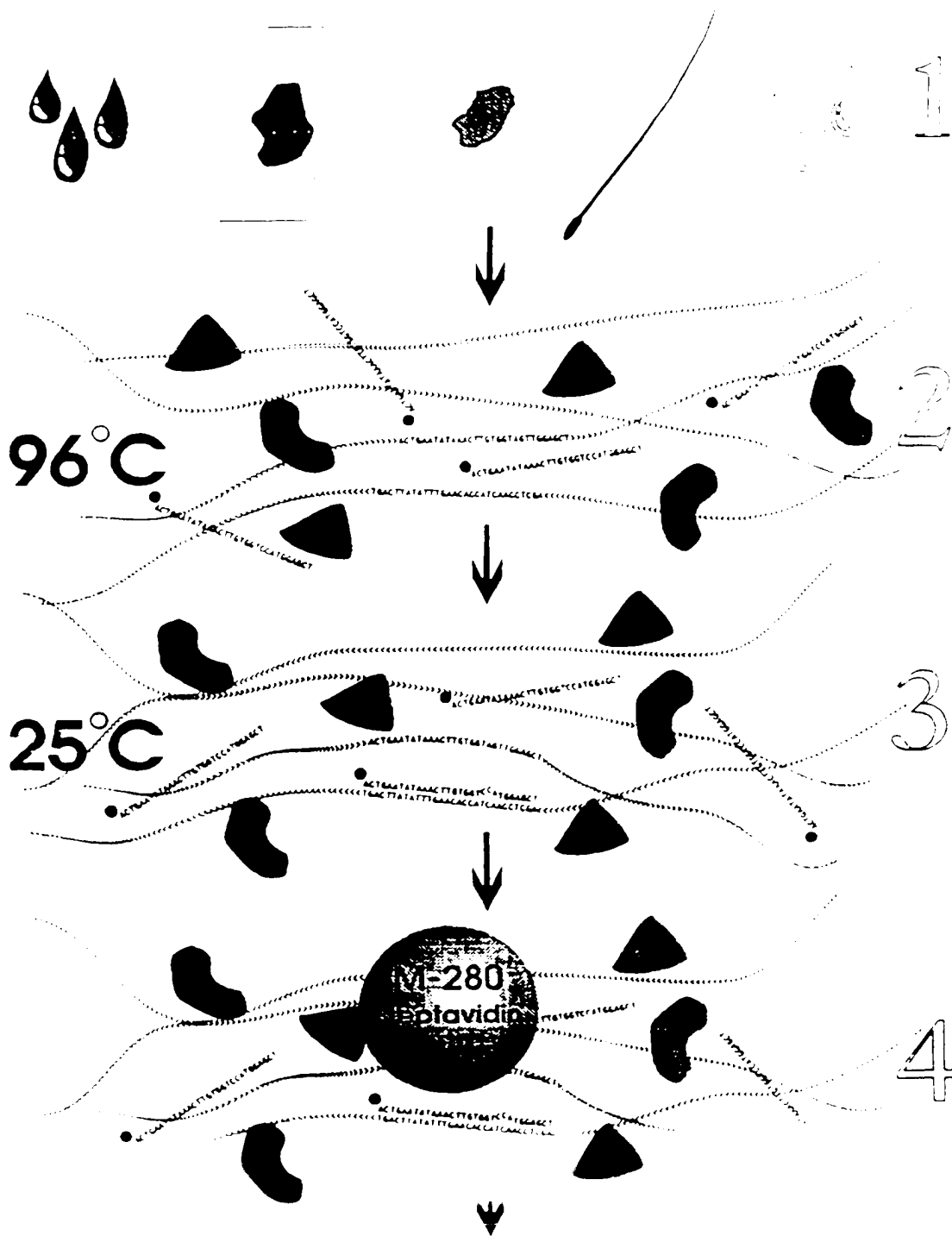
d) Q-beta replicase and the kit additionally comprises an RNA directed RNA polymerase and an RNA probe with a 5'-MDV-1 structure, the capture oligonucleotide being immobilised or permitting immobilisation.

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g Glu Gln Ile Lys Arg Val
 TTTGAAAGATATTTTCTTACTAATGACTGTGTATATAATTTTTTTTTTCTCCAG A GAA CAA ATT AAA AGA GTT
 EXON 1 -
 Lys Asp Ser Glu Asp Val Pro Met Val Leu Val Gly Asn Lys Cys Asp Leu Phe Ser Arg Thr
 AAG GAC TCT GAA GAT GTA CCT ATG GTC CTA GTA GGA AAT AAA TGT GAT TTG CCT TCT AGA ACA
 Val Asp Thr Lys Gln Ala Gln Asp Leu Ala Arg Ser Tyr Gly Ile Pro Phe Ile Gly Thr Ser
 GTA GAC ACA AAA CAG GCT CAG GAC TTA GCA AGA AGT TAT GGA ATT CCT TTT ATT GAA ACA TCA
 Ala Lys Thr Arg Gln
 GCA AAG ACA AGA CAG GTAAGTAACACTGAAATAAATACAGATCTGTTTTCTGCAAAATCATAACTGTTATGTCATTTA
 EXON 1
 ATATATCAATTTTTCTTCAATTATGCTATAGTAAATAAAAACAATATTTAGTAAATGTTTTTGTCTCTTGAGAGGGCATTG
 GTTCTTAATC>>>ACAGAAGACCCAGTCTCAGCTTCAGTTGTATACCTGGAAATAGACTGAAAGGTCTTAAATTTTAAATAA
 AACTCAAGGTTCCAGTTTTCTTCACTCAGCTTTGAGATTTCTTATGTTTTTGTGTTTTTAAACAAGGTTTCAGCTCCATATT
 TTACCATTTTTCTCTCATTCTCCCTGGAGGAGGGGTGTGGGAATCGATAGTATATAAATCACTTTTTCTCAAGTCAAGAA
 GTAATTTAAAGCTAACTTCAGTTTAGGTTTTAATTCAGGACTAGCAAACTAAATGGTTGCATTAAATGCAAAACAGATGCTA
 ATACCTCTCTTTAGGCTTGTATATCTCTCTTAATTTCTAATTTTAAATAATTTTAAATTTTAAATTTTAAATTTTAAATTT
 GACTTTTAAAGAACAAACAGGATTCTAGGCTATATTTTAAACTGCATCTCAGTTTTATTCAAACAGTCTGATGTCTGTTTAA
 AAAAAAAAAAATCTCAAGCTCATAATCTCAAACTTTTGCACATGGCTTTCCAGTAAATTACTCTTATCAATGCAACAGACT
 Arg Val Glu Asp Ala Phe Tyr Thr Leu Val Arg Glu Ile Arg
 TTAAAGAAGTTCTGTTTTTACAATGCAG AGA GTG GAG GAT CCT TTT TAT ACA TTG GTG AGA GAG ATC CGA
 EXON 1A -
 Gln Tyr Arg Leu Lys Lys Ile Ser Lys Glu Glu Lys Thr Pro Gly Cys Val Lys Ile Lys Lys
 CAA TAC AGA TTG AAA AAA ATC AGC AAA GAA GAA AAG ACT CCT GGC TGT GTG AAA ATT AAA AAA
 Cys Ile Ile Met OC
 TGC ATT ATA ATG TAA TCTG GTAAGTTTAAGTTCAGCACATTAATTTTGGCAGAAAGCAGATGCTTTTTAAAGGTAACA
 EXON 1A
 AGGTGGCAACCACITTAGAAGTACTTAGGTGTAGTATTCTAAGTTGAAGTATTAAGATAAGAACTTGTCTTCCATAATTAG
 T>>>GAATTTTAAAGTCTTAATATATATATATATATATTCAGTTGCTGGAAGAGAAACATAAAGAATCCTTTCTTAATTTTTT
 TCCATTAATGAAATTTGTTACCTGTACACATGAAGCCATCGTATATATTCACATTTTAATACTTTTTATGTATTTTCAAG
 Gly
 EXON 1A
 Val Asp Asp Ala Phe Tyr Thr Leu Val Arg Glu Ile Arg Lys His Lys Glu Lys Met Ser Lys
 GTT GAT GAT GCC TTC TAT ACA TTA GTT CGA GAA ATT CGA AAA CAT AAA GAA AAG ATG AGC AAA
 Asp Gly Lys Lys Lys Lys Lys Lys Ser Lys Thr Lys Cys Val Ile Met OC
 GAT GGT AAA AAG AAG AAA AAG AAG TCA AAG ACA AAG TGT GTA ATT ATG TAA ATACAATTTGTACTT
 TTTTCTTAAGGCATACTAGTACAAG TGGTAATTTTTGTACATTACACTAAATTATTAGCATTTGTTTTAGCATTACCTAATTT
 EXON 1A
 TTTTCTGCTCCATGCAGACTGTTAGCTTTTACCTTAAATGCTTATTTTAAATGACAGTGGAGTTTTTTTTCTCGAAGT
 GCCAGTATTTCCAGATTTTTGGTTTTTGAAGTACCAATGCCTGTGAAAAAGAACTGAATACCTAAGATTCTGTCTTGGGGTT
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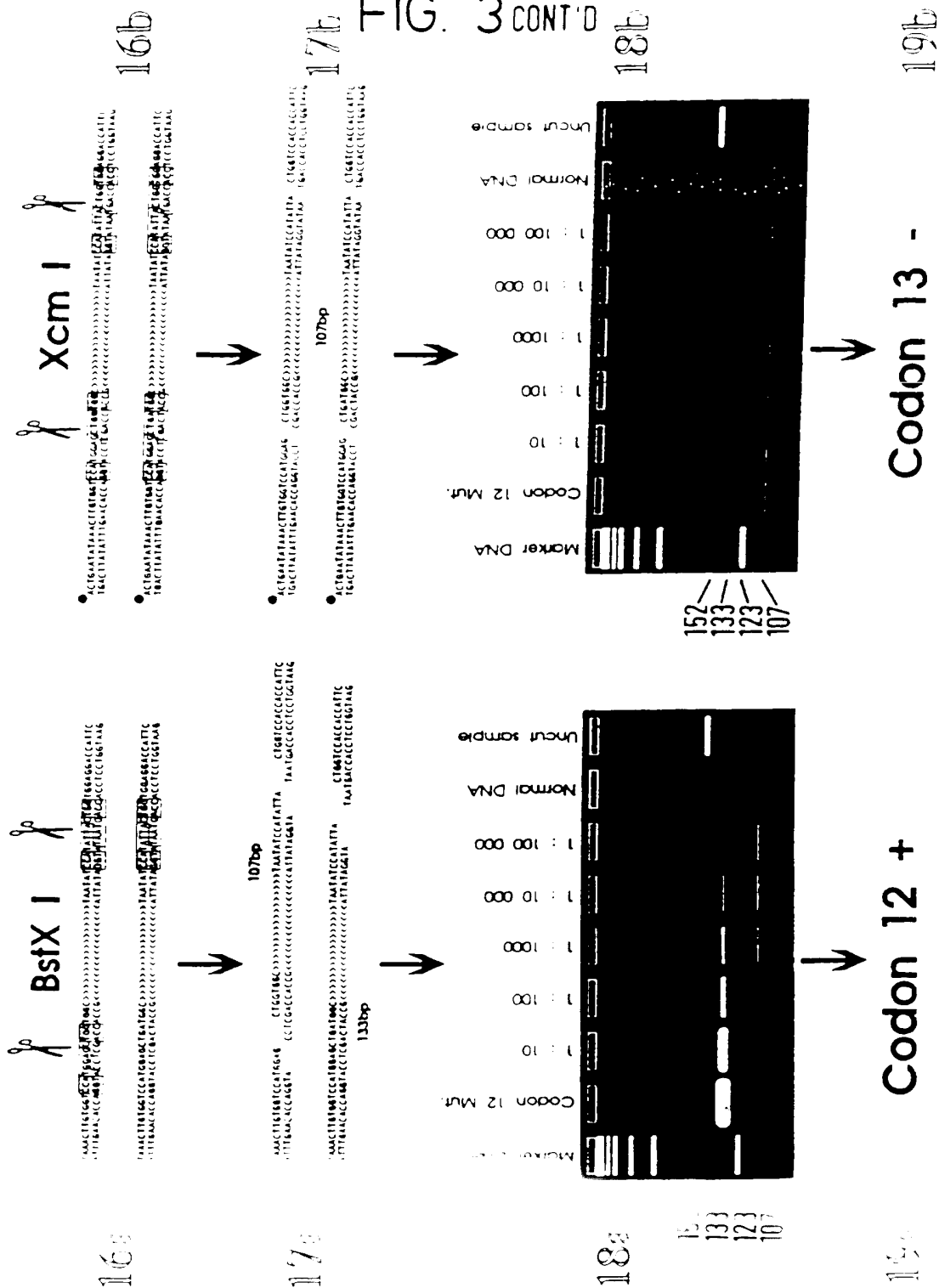
FIG. 1CONT'D

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FIG. 3

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FIG. 3 CONT'D



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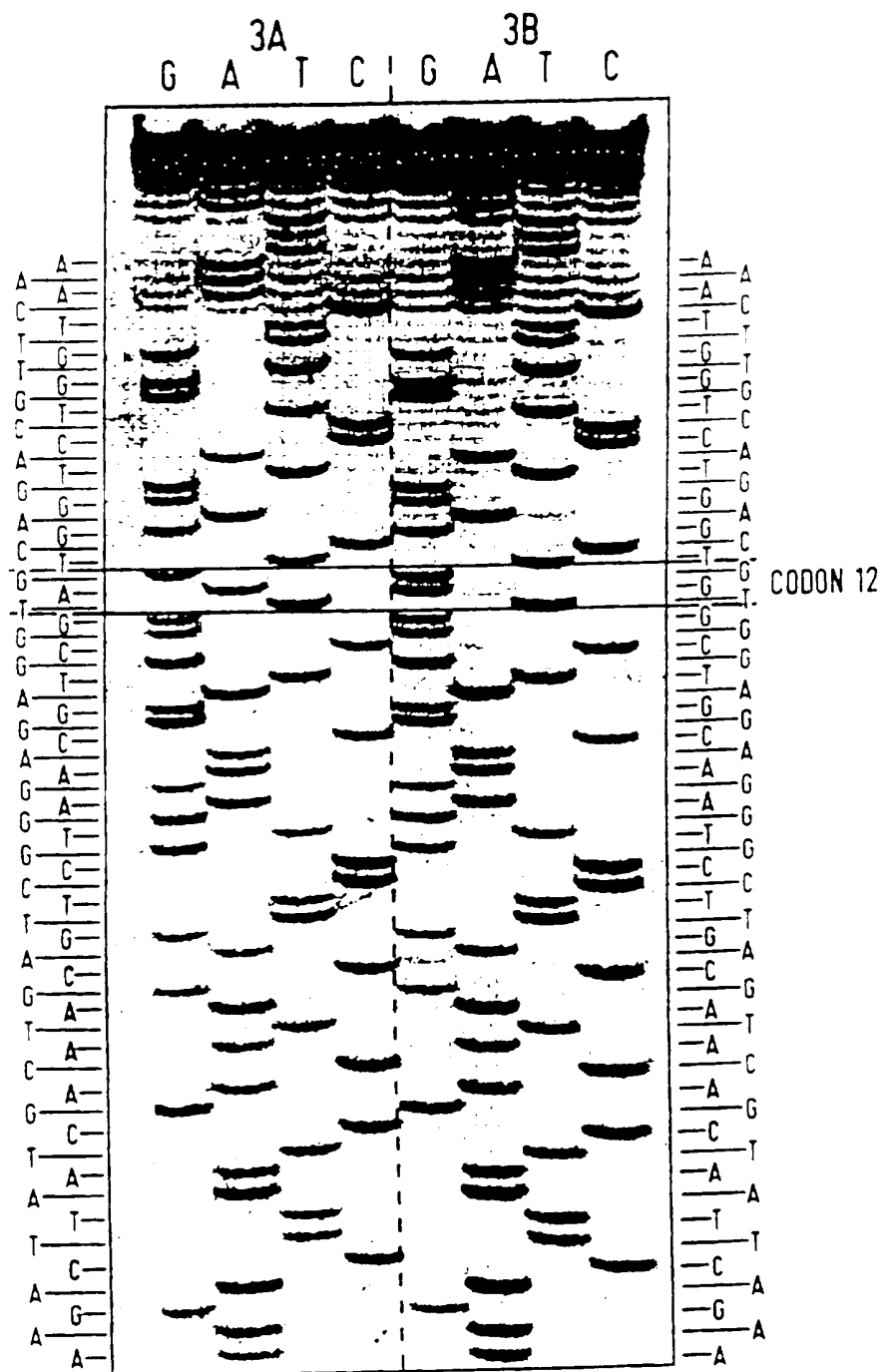


FIG. 5

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(54) Title: METHOD FOR THE DETECTION OF RAS ONCOGENES, IN PARTICULAR THE 12-RAS ONCOGENE

(57) Abstract

The invention relates to an oligonucleotide primer sequence for use in *in vitro* amplification, characterised in that said primer sequence is capable of creating a *Bst*X I restriction site overlapping codon (12) and/or an *Xcm* I restriction site overlapping codon (13) or a *Bce* 831 I restriction site overlapping codon (61) of the wild-type *K-ras* oncogene, methods of using said primer sequences for detecting activating mutations in codons (12 and/or 13 and/or 61) of the *K-ras* oncogene and kits for performing the methods.

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A. CLASSIFICATION OF SUBJECT MATTER
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AM. J. CLIN. PATHOL. vol. 100, no. 6, December 1993, pages 686-89, XP000572107 LIN S ET AL: "mutation analysis of K-ras oncogenes in gastroenterologic cancers by the amplified created restriction sites method" see the whole document ---	1-22
A	ONCOGENE, vol. 6, no. 8, 1991, pages 1353-62, XP002004427 MITSUDOMI T ET AL: "Mutations of ras genes distinguish a subset of non-small-cell lung cancer lines from small-cell cancer cell lines" see the whole document ---	1-22
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